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Effect of Thrombin on Maturing Human Megakaryocytes

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Thrombin causes platelet activation and secretion. In some nucleated cells, it is mitogenic. In this study, we have investigated how human megakaryocytes (MKs) respond to this agonist and whether the response depends on the maturation stage. MKs were cultured from bone marrow precursors in liquid culture in the presence of normal plasma. To determine whether thrombin can activate MKs, 14-day MK cultures were incubated with thrombin for 5 minutes, and cells were studied by electron microscopy, either by standard techniques or after embedding in glycolmethacrylate for immunoelectron microscopy. Ultrastructural examination of thrombin-treated MKs revealed dramatic morphological changes reminiscent of those found in platelets, including shape change and organelle centralization that involved immature as well as mature cells. MKs were also able to secrete α-granule proteins in the dilated cisternae of the demarcation membrane system, as shown by immunogold staining for thrombospondin and glycoprotein Ib. These changes were rapid (less than 5 minutes) but despite them, MKs remained viable for more than 24 hours. To determine whether thrombin has a mitogenic activity, it was added to the culture of MKs from day 3 to day 10 of culture at concentrations varying from 0.1 to 10 U/ml. Cells were subsequently studied by a double staining technique using flow cytometry to determine MK number and ploidy. No changes were observed in these two parameters, showing that thrombin is not mitogenic for MKs at the concentrations used. In conclusion, this study confirms for human MKs previous observations made about guinea pig MKs (Fedorko et al, Lab Invest 1977, 36:32). In addition, it demonstrates that immature MKs are able to respond to thrombin and that more mature cells can secrete α-granule proteins into the demarcation membrane system, which is in continuity with the extracellular space. This phenomenon may have implications for pathological states such as myelofibrosis formation and for megakaryopoiesis autocrine regulation. (Am J Pathol 1993, 143:1498–1508)

Megakaryocytes (MKs), the bone marrow precursors of blood platelets, are mainly located along the walls of the bone marrow sinusoids during maturation.[1] They are able to cross this barrier and enter the circulation when they become mature.[2] MKs give rise to platelets by fragmentation of their cytoplasm into a multitude of anucleated subunits. MKs have much in common with platelets, including receptors, plasma membrane components, and storage proteins.[3] The serine protease thrombin is a multifunctional key enzyme in coagulation, formed at the site of vascular injury with various biological effects: it is a potent agonist of platelet activation, eg, it activates G proteins, increases calcium and pH levels, leads to activation of phosphokinase C, phospholipase A2, and tyrosine kinases, and inhibits adenylate cyclase;[4] it might also play an important role in vascular repair.[5] It was reported to be a potent mitogen of diverse cell types such as fibroblasts, lymphocytes, and mesenchymal cells,[6,7] to be chemotactic for monocytes,[8] and to induce the expression of P-selectin on endothelial cells[9] and the production of platelet-derived growth factor.[10] Therefore, thrombin activates platelets and several other cell types causing either changes in their functional state or mitotic events.

Recently, the functional thrombin receptor has been cloned and its messenger RNA was detected in

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platelets and in the megakaryocytic cell lines, Dami and HEL, suggesting that it is also expressed in normal MKs.[11] In this report, we investigated the effects of thrombin on human cultured MKs. Because MKs share antigenic components with platelets including glycoprotein (GP) Ib, which can bind thrombin,[12] we investigated both the activating and mitogenic effects of thrombin on human cultured MKs.

Materials and Methods

Cell Samples

Bone marrow samples were aspirated from donors for bone marrow transplantation after informed consent. They were collected into sterile syringes containing preservative-free heparin (50 U/ml) (Roche, Paris, France). Low-density cells (<1.077 g/ml) were obtained by Ficoll-metrizoate (Biochrom KG, Berlin, Germany) gradient density centrifugation and were washed three times in α medium (Eurobio, Paris, France) containing penicillin, streptomycin, and glutamine. Adherent cell depletion was performed by incubation of the cell suspension (1×10^6 per ml) in α medium with 10% fetal calf serum (Gibco, Paisley, Scotland) for at least 1 hour on plastic culture Petri dishes. Nonadherent cells were recovered and used for cell cultures in liquid medium. All these samples were obtained in accordance with the guidelines of Hospital Ethics Committee.

Megakaryocyte Liquid Culture

Low-density cells were cultured for 12 days as described previously.[13] Briefly, Iscove's modified Dulbecco's medium (Gibco, Paisley, Scotland) was supplemented with 1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) and 10% serum derived from plasma from patients with aplastic marrows (thrombocytopenic patients after a bone marrow transplantation). Cell cultures were performed for 12 days in 25-cm^2 or 75-cm^2 tissue culture flasks (Corning Glasswork Co., New York, NY) at 37 C in a 5% CO_2 fully humidified atmosphere at 1×10^6 cells par ml. Purified human thrombin (Sigma) (0.1 U/ml and 0.5 U/ml) was added once, either at day 0, 5, 7, or 9 of culture.

Electron Microscopy (EM)

MKs from liquid culture were washed twice in the presence of prostaglandin E1 (Sigma) and apyrase, resuspended in Tyrode's buffer containing 2.9 mmol/L CaCl2 and exposed to α-thrombin 0.1 U for 5 minutes. The reaction was stopped, and MKs were fixed in 1% glutaraldehyde in Tyrode's buffer, washed three times in phosphate buffer, and embedded either in Epon for standard EM examination, or in glycol methacrylate for immunoelectron microscopy. For immunolabeling, thin sections were incubated facing down on microwells containing polyclonal antibodies directed against GPIb[14] and/or thrombospondin[15] followed by goat anti-rabbit immunoglobulins coupled to 10-nm colloidal gold (Amersham les Ullys, France). The sections were observed on a Philips CM10 electron microscope.

Megakaryocyte Ploidy Measurement

A double staining technique was used to measure the ploidy of MKs. Cells in the liquid culture system (at day 10 of culture) were fixed by 2% paraformaldehyde (Serva, Heidelberg, Germany) for 20 minutes at 4 C and washed three times in phosphate-buffered saline solution. A monoclonal antibody directed against GPIIIa (Y2/51) was used to identify MKs, followed by fluorescein-conjugated goat immunoglobulin G directed against mouse immunoglobulin (Silenus, Hawthorn, Australia). Cells were washed in phosphate-buffered saline and permeabilized by 0.002% Triton X-100 (Sigma) for 20 minutes. A propidium iodide (Sigma) solution at 50 mg/ml in isotonic sodium citrate containing 100 mg/ml RNAse (Boehringer, Mannheim, Germany) was used for DNA staining. Cells were analyzed by an ATC 3000 flow cytometer (Bruker, Wissembourg, France).[13] The cell flow rate was 1,500 cells per second. The frequency of MKs in different classes was evaluated usually on 10,000 MKs. All ploidy classes over 16N were counted together because these MKs were rare in culture.

Megakaryocyte Colony Assay

The plasma clot technique was used as previously described.[16,17] Normal serum was used at a 10% concentration. Cells were plated at 1.5×10^5 cells per ml in triplicate in the presence or the absence of 100 U/ml recombinant interleukin-3 (IL-3) (Genetics Institute, Cambridge, MA). In the assays, thrombin was added at 0.1, 0.5, 1, and 10 U/ml. At day 12, MKs were stained *in situ* by indirect immunofluorescence, using the Y2/51 monoclonal antibody.

Each dish was entirely scanned under an inverted microscope equipped for epifluorescence.

Results

Electron Microscopy

Immature MKs

Immature MKs could be distinguished from other cells by their relatively large size, high nuclear to cytoplasmic ratio, and nuclear indentation. Some of these immature MKs were forming demarcation membranes and α-granules (Figure 1a). On the very immature cells, thrombin induced membrane ruffling, nuclear condensation, and formation of nuclear blebs (Figure 1b). Immunoelectron microscopic detection of thrombospondin and GPIb performed on MKs at an intermediate maturation stage showed that some dense material composed of released α-granule proteins, such as thrombospondin (Figure 2a) was present in the forming demarcation membrane system lined by GPIb (Figure 2b).

Mature MKs

Mature MKs were recognized by their large size and cytoplasm containing numerous demarcation membranes and α-granules (Figure 3a). After thrombin was applied to the cells, dramatic morphological changes were observed: membrane ruffling with the formation of numerous long pseudopodia (Figures 1b and 4a) and the appearance of a submembranous network of demarcation membranes (Figure 3b). The nucleus often became eccentric (Figures 3b and 4b) with centralization of the other organelles, granules, and demarcation membranes, surrounded by a dense filamentous knot resembling the one observed in activated platelets (Figure 3b). The cisternae of the demarcation membrane system became widened and filled with dense material. Simultaneously, α-granules were more seldom in the cytoplasm. In our experimental conditions (no agitation), no aggregation of mature MKs was observed. Immunolabeling for thrombospondin (Figure 5a) confirmed that the dense material filling the dilated channels of the demarcation membrane system originated from α-granules, and the observation of GPIb labeling lining them (Figure 5b) confirmed that it was indeed contained within the demarcation membranes. Alpha-granules were only occasionally found in the cytoplasms of activated MKs.

Effects of Thrombin on the Proliferation of CFU-MK and MK Endomitosis (Figure 6)

In a first set of experiments, thrombin (0.1, 0.5, 1, and 10 U/ml) was added at the onset of the cultures in the colony-forming unit-MK assay. No effect was observed on the number of MK colonies when MK colonies were grown either in the absence of any added growth factor or in the presence of IL-3 (Figure 6a).

In a second set of experiments, the effect of thrombin was tested in a liquid culture technique associated with flow cytometry, which allowed to measure the number of MKs obtained in culture from the differentiation of colony-forming unit-MK (mitosis) and the ploidy of these cultured MKs (endomitosis). Thrombin was added at several concentrations (0.1 to 10 U/ml) at different days of culture. Indeed, in this culture system, polyploidization of MKs begins at day 6 of culture, and no differences in the ploidy distribution are observed after day 9. We did not find any marked differences in the number and ploidy of MKs after thrombin addition. Five tenths, 1, and 10 U/ml of thrombin gave results which were not significantly different from 0.1 U/ml (not shown). A typical experiment is shown in Figure 6b. However, these experiments were performed three times and the differences in the number of MKs observed and the ploidy distribution as calculated by the Student *t*-test were not significant.

Discussion

In this study, we have investigated the responsiveness of human MKs to thrombin stimulation. A functional thrombin receptor has been cloned and found to be present on platelets, vascular endothelial cells, and the MK-like cell lines Dami and HEL.[11] It was also demonstrated that MKs were cells able to be activated and respond to various stimuli such as adenosine diphosphate, thrombin, arachidonic acid, and collagen by platelet-like morphological and metabolic changes. These include thromboxane synthesis, shape change, and cytokeletal alterations.[18–25] Thrombin has also a potent biological mitogenic effect on other cell types such as lymphocytes,[7] endothelial cells,[26–28] fibroblasts,[29–31] and smooth muscle cells.[32] Recently, thrombin was shown to inhibit proliferation of a human megakaryoblastic cell line MEG 01, this effect involving a possible cyclic Adenosine monophosphate-dependent mechanism.[33] Thus, in this study, both the effects of thrombin on activation and mitosis of human cultured MKs were explored.

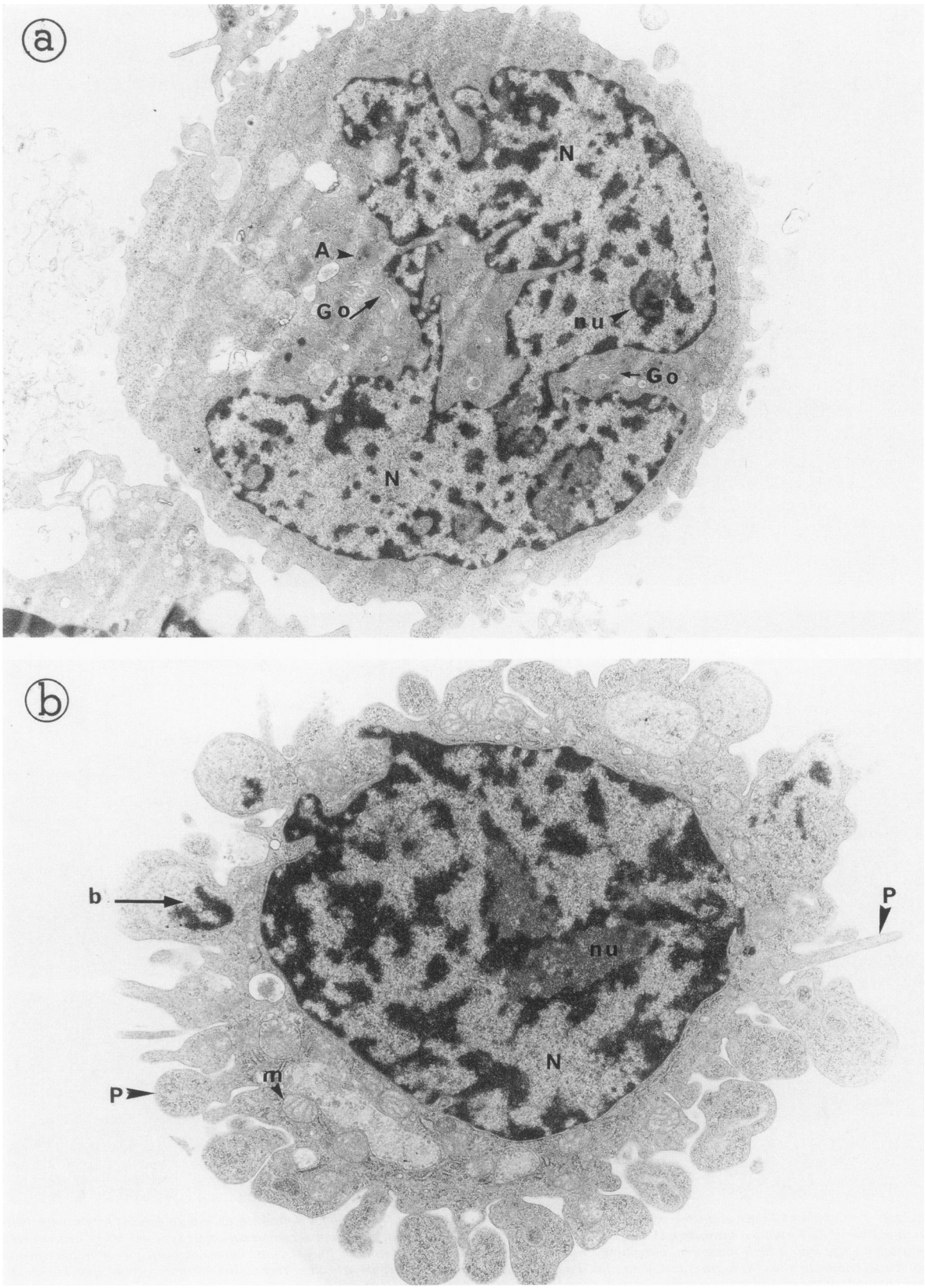

Figure 1. a: *The EM appearance of this early stage of MK maturation is characterized by a moderate cell size, high nuclear to cytoplasmic ratio, rarity of α-granules (A) and prominent nucleolus (nu). The cell surface is relatively smooth and the nucleus (N) is centrally located (Go: Golgi) (6,785×).* b: *Thrombin stimulation (0.1 U/ml) of this immature MK causes nuclear bleb formation and the appearance of pseudopods (P) on the cell surface (10,250×).*

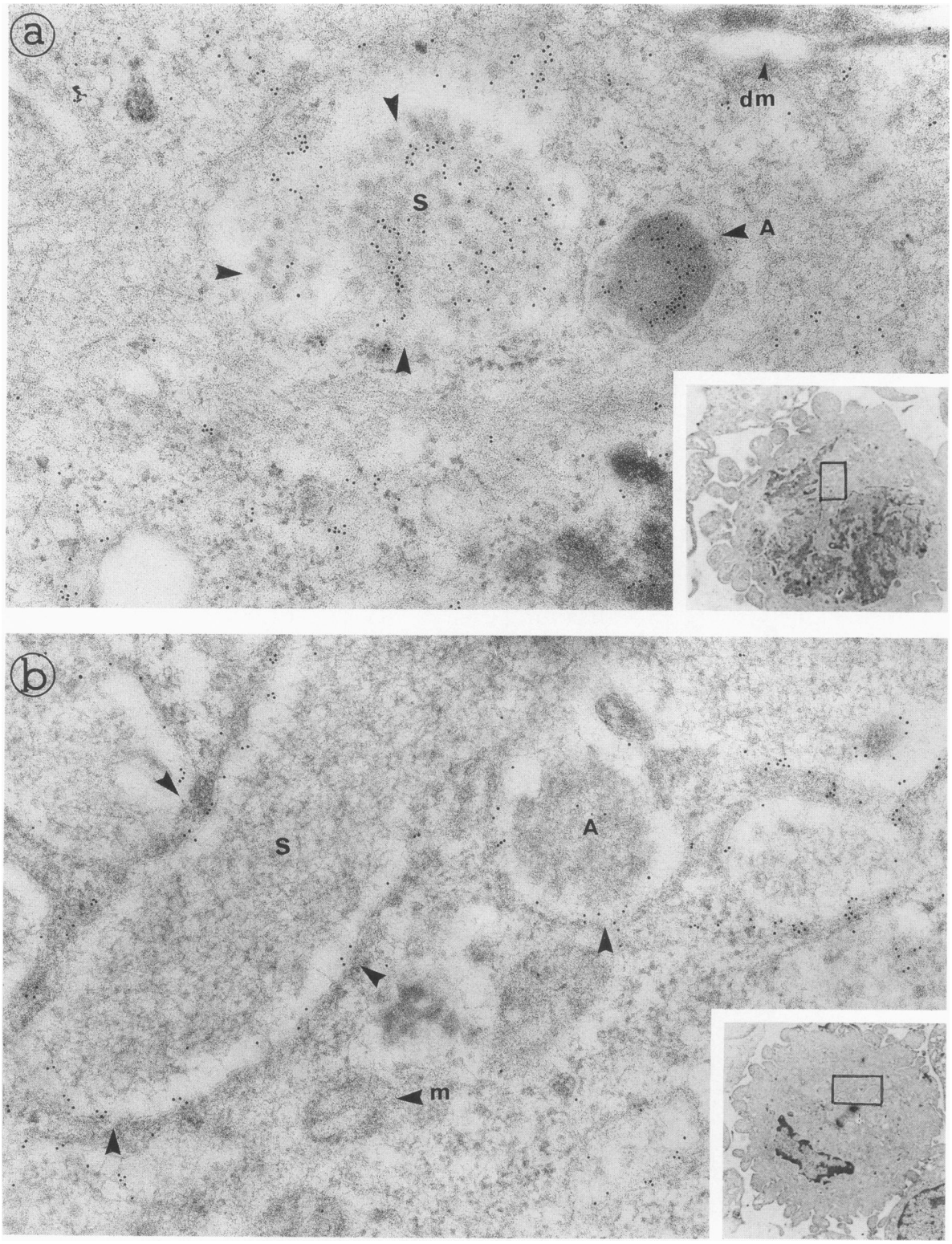

Figure 2. *Part of a middle size maturing megakaryocyte stimulated by thrombin and 0.1 U/ml for 5 minutes.* a: *Immunolabeled for thrombospondin, a marker of the forming α-granules (A): the antigen is redistributed to some intracytoplasmic secretion vacuoles (S) of this MK of intermediate maturation stage with a high nuclear to cytoplasmic ratio (dm: demarcation membranes) (36,800×).* b: *Immunolabeled for GPIb: after fusion with the secretion vacuoles (S) α-granules (A) are lined by immunolabeling for GPIb (arrowheads). These labeled structures correspond to the dilated channels of the demarcation membrane (dm) system (36,800×).*

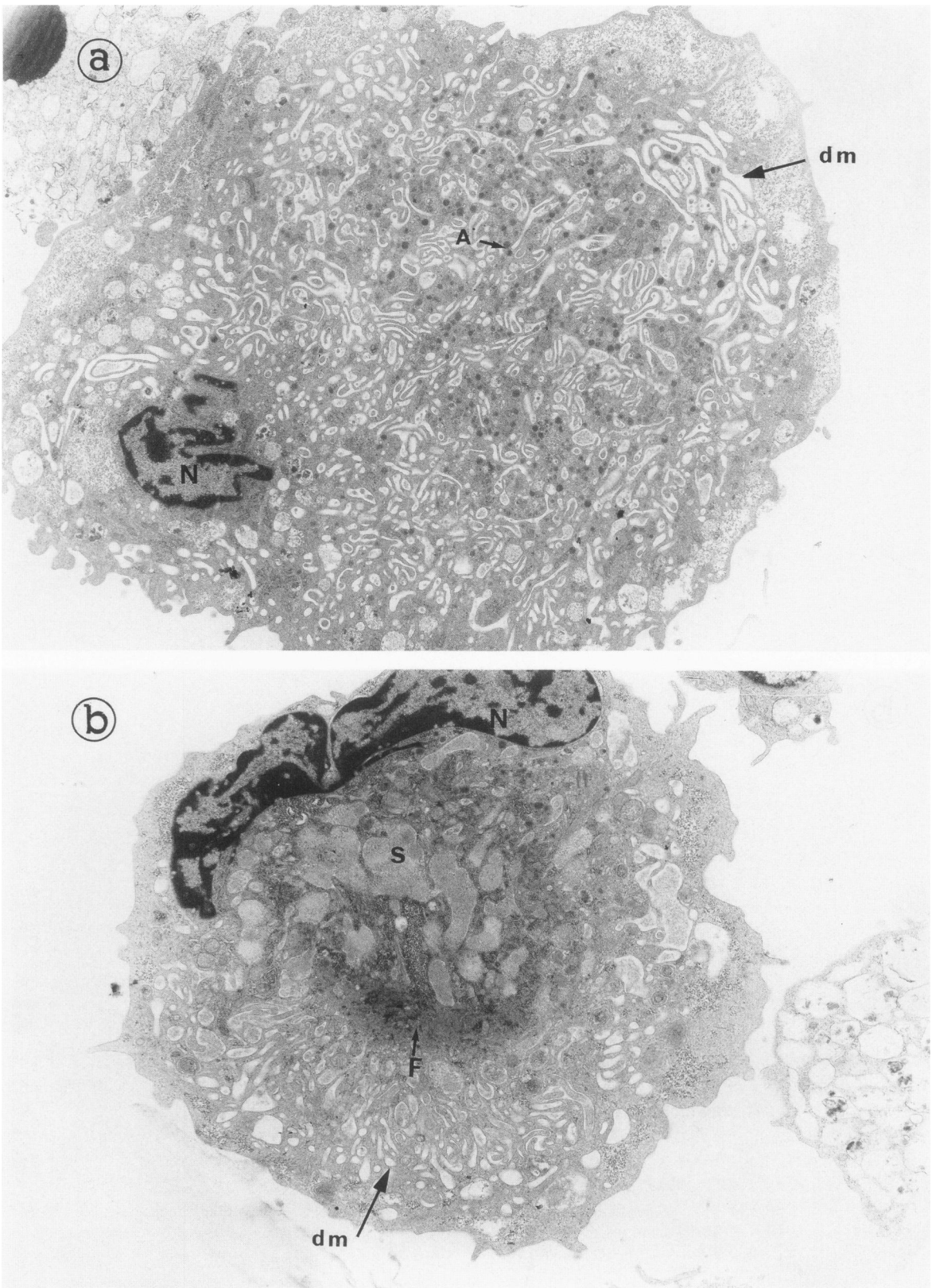

Figure 3. a: *This control mature MK appears very large with low nuclear to cytoplasm ratio and has numerous α-granules (A) and prominent demarcation membrane system (dm) whose lumen is clear. The cell surface is relatively smooth (4,700×).* b: *In some thrombin-treated (0.1 U/ml) mature MKs, an eccentration of the nucleus (N) is observed that surrounds concentric layers: a demarcation membrane zone (dm), a ring of dense filamentous meshwork (F), and a central zone containing some dilated cisternae of the demarcation membrane system filled with secretion material (S) and a few α-granules (A) (6,200×).*

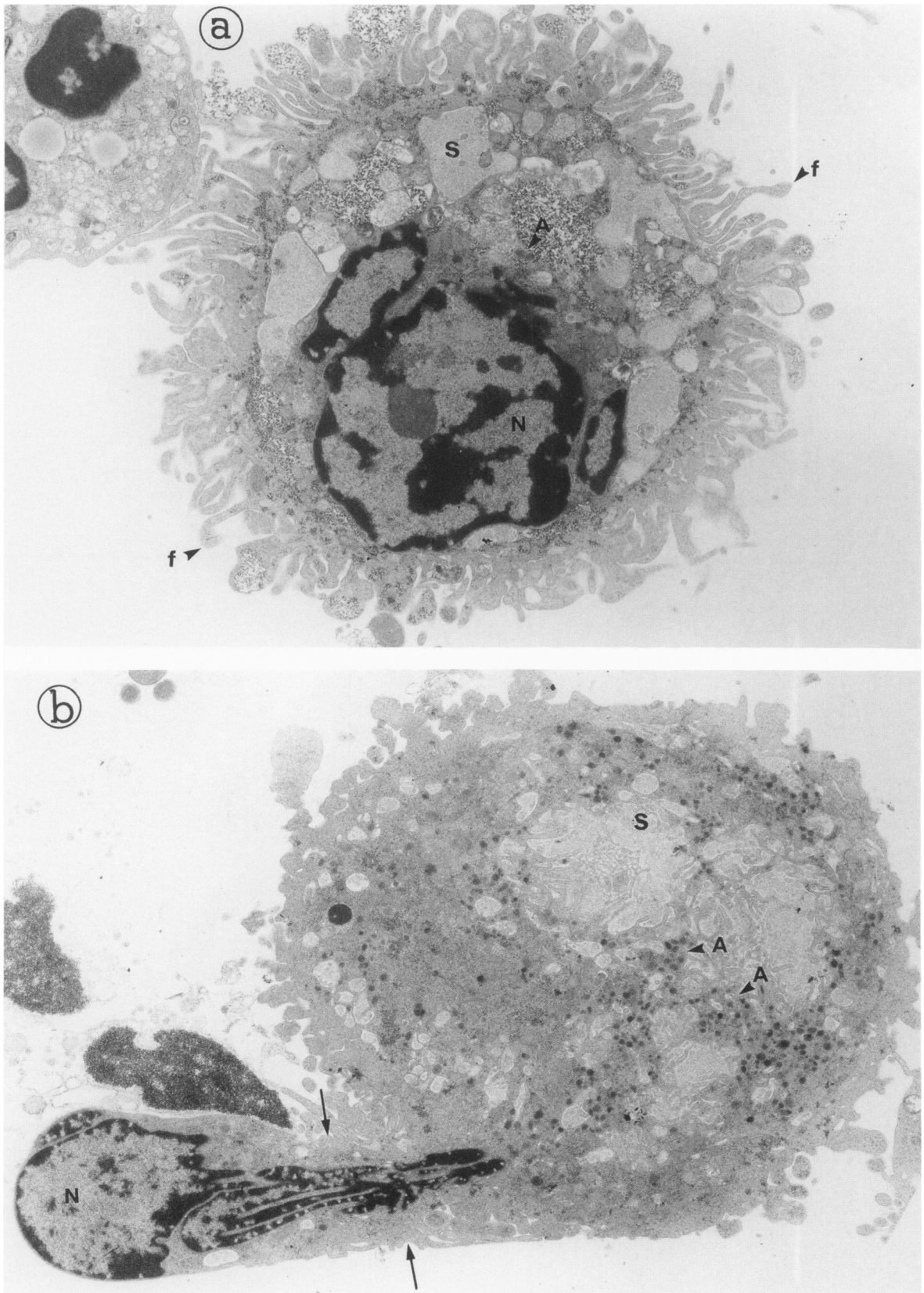

Figure 4. a: *Thrombin stimulation (0.1 U/ml) of the mature cultured MK enhances the emission of long and thin pseudopods (filopodia) (f), dilatation of the demarcation membranes and concomitant secretion (S): only a few α-granules (A) remain in the cytoplasm (7,800×).* b: *Some mature MKs display a very eccentric nucleus located at one pole of the cell, almost on the process of being shed (arrowheads) from the voluminous cytoplasm. Prominent dilatation of demarcation membranes forming secretion vacuoles (S) is observed (A: α-granules) (4,800×).*

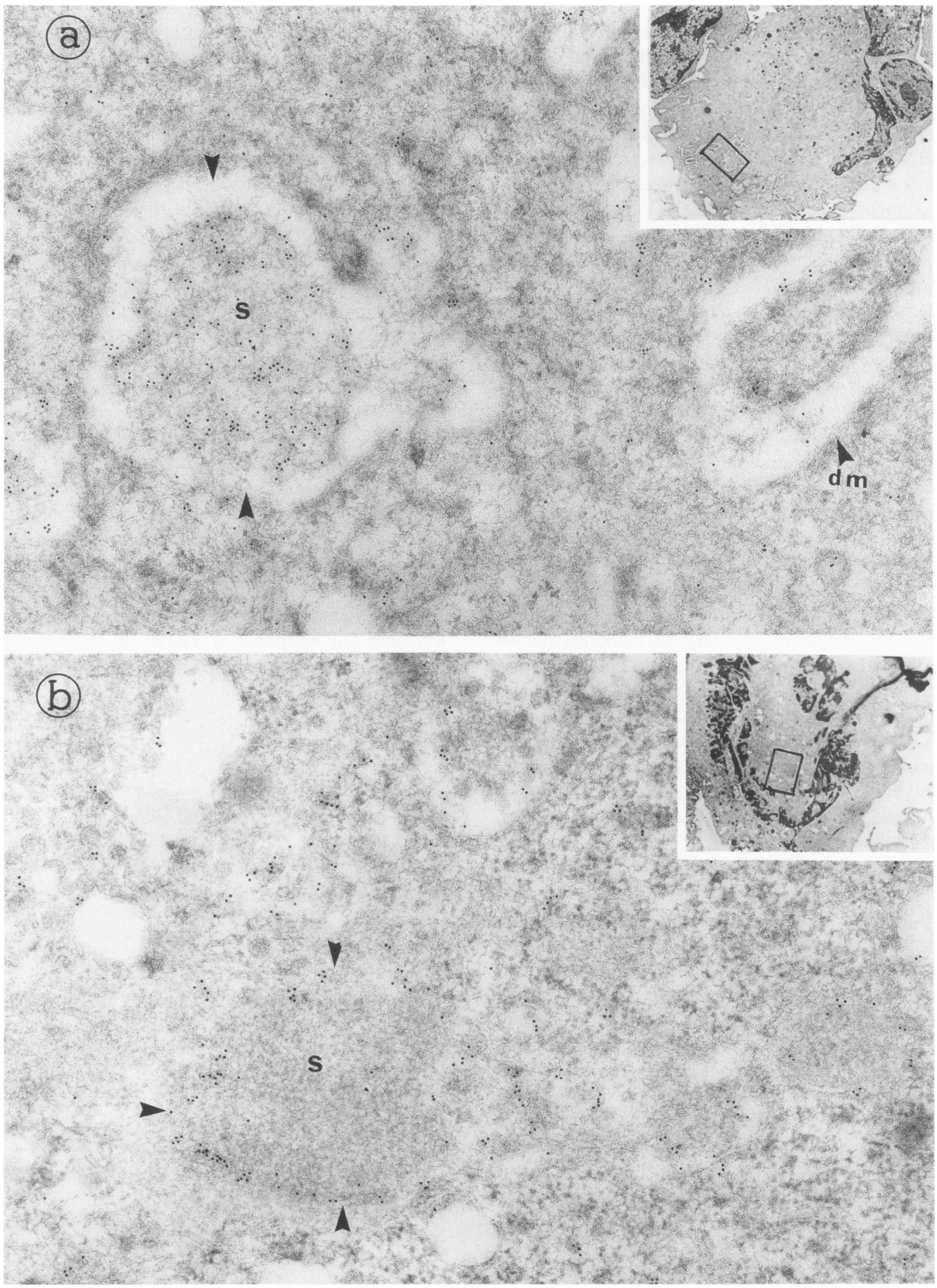

Figure 5. *Part of a large, fully mature MK stimulated by thrombin (0.1 U/ml).* a: *Immunolabeled for the α-granule protein thrombospondin: the protein is found in the large areas of secretion (S) formed by the fusion of some α-granules with the demarcation membranes (dm) (36,800×).* b: *Immunolabeled for GPIb: the large secretion areas (S) are limited by the dilated channels of the demarcation membrane system because they are lined by membrane-specific labeling (arrowheads) (36,800×).*

On the one hand, we have shown that cultured human MKs, mature as well as immature cells, strongly react to thrombin by dramatic morphological changes, including some resembling the effects of thrombin on platelets: membrane ruffling and extensive formation of pseudopods around the cell surface and/or eccentric positioning of the nucleus at one pole of the elongated cytoplasm; evidence of contractility with a thickening of the marginal zone and centralization of organelles, including granules and dilatated cisternae of the demarcation membrane system and the appearance of a central, dense filamentous knot; and secretion of the α-granule content within the demarcation membrane system. The formation of nuclear blebs in the immature MKs reminded us of the nuclei of leukemic cells from megakaryoblastic leukemia (M 7) which often exhibit this peculiar and specific abnormality.[34] No spreading of MKs was obtained in our experimental model, unlike what Leven and Nachmias had observed in their study;[25] however, they used a higher dose of thrombin (1 U/ml), and full MK spreading was only observed after a long incubation time (30 minutes). Also they studied mature bone marrow MKs instead of MK cultures from progenitors. The nature of contractile response that we observed seems to differ from one cell to another, membrane ruffling (Figures 1b and 4a) and actomyosin centralization (Figure 3b). It is a possibility that these phenomena are sequential: first membrane ruffling, followed by contraction, secretion, and then by smoothing of the cell surface. Previous studies, performed on guinea pig MKs had shown that MKs could react to some platelet agonists by several structural modifications.[18,25] The originality of our study is that it is the first to deal with human MKs and to show that not only mature but also immature MK cells can respond to thrombin. Also the use of immunoelectron microscopy has allowed us to show that thrombin induces a fusion of the α-granules with the demarcation membrane system and the subsequent secretion of granule content into the demarcation membrane channels which are in continuity with the extracellular space. It would be of interest to know whether this phenomenon is relevant in the development of myelofibrosis, as α-granules contain platelet-derived growth factor, which stimulates fibroblast growth and synthesis.[35–37] It may also be involved in autocrine regulation of megakaryocytopoiesis by the release of platelet factor-4, β-thromboglobulin, and transforming growth factor-β, which are natural inhibitors of megakaryopoiesis.[38–40] MKs have been demonstrated to be present in large numbers in the central venous circulation,[41] and evidence has also been advanced that they fragment into platelets in the lungs.[42] It is therefore possible that the MK secretion properties described here may also be involved in pulmonary disease such as lung fibrosis.[43] Therefore, it might be of interest to try to develop means of controlling their possible activation.

On the other hand, our results fail to demonstrate any effect of thrombin on MK colony formation or MK ploidy, suggesting that thrombin has no mitogenic activity on either early or late stages of the MK differentiation at the concentrations used here. Although thrombin is able to activate MKs, it has no clear mitogenic effect on the MK lineage. Inactivation of thrombin by the added serum is unlikely because, even at low dose (0.1 U/ml), it could induce a strong activation of MKs as demonstrated by the ultrastructural changes. Moreover, IL-3 added in the same experimental conditions induced an increase of colony-forming unit-MK, demonstrating that the precursors were not beyond the stage of mitosis. The discrepancy between these two thrombin effects may be related to a late expression of the thrombin receptor at a differentiation stage where MK cells are incapable of mitosis or endomitosis. Alternatively, the transduction signal induced by the

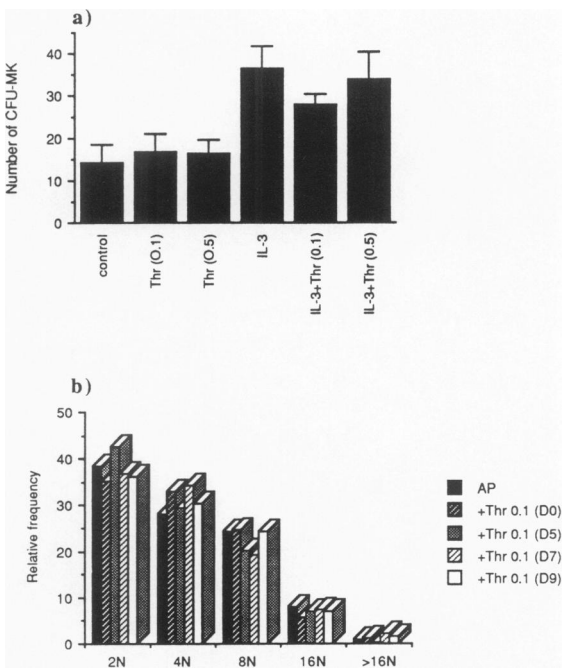

Figure 6. a: *Effect of thrombin (0.1 and 0.5 U/ml) and IL-3 on the number of MK colonies.* b: *Effect of thrombin (0.1 and 0.5 ml) on the cultured MK ploidy.*

binding of thrombin to its receptor may cause functional response but no proliferation per se in some cell types such as MKs, monocytes, or endothelial cells.[44–45] The mitogenic effect of thrombin may only be effective on G0 cells and not on cycling cells such as hematopoietic cells. Future work investigating the expression of the thrombin receptor during differentiation may be helpful to understand better these differences in the action of thrombin on MKs.

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